

Video Article

# ***In Vitro* Method to Control Concentrations of Halogenated Gases in Cultured Alveolar Epithelial Cells**

Raïko Blondonnet<sup>1,2</sup>, Bertille Paquette<sup>1,2</sup>, Damien Richard<sup>3</sup>, Rémi Bourg<sup>2,4</sup>, Géraldine Laplace<sup>2,4</sup>, Romain Segurel<sup>2,4</sup>, Henria Pouvelle<sup>2,4</sup>, Corinne Belville<sup>2</sup>, Loïc Blanchon<sup>2</sup>, Thomas Godet<sup>1,2</sup>, Jean-Michel Constantin<sup>1,2</sup>, Jean-Etienne Bazin<sup>1,2</sup>, Vincent Sapin<sup>2</sup>, Matthieu Jabaudon<sup>1,2</sup>

<sup>1</sup>Department of Perioperative Medicine, CHU Clermont-Ferrand

<sup>2</sup>Centre National de la Recherche Scientifique Unité Mixte de Recherche (CNRS UMR) 6293, Institut National de la Santé et de la Recherche Médicale (INSERM) U1103, Laboratoire de Génétique, Reproduction et Développement (GRoD), Université Clermont Auvergne

<sup>3</sup>Department of Pharmacology, CHU Clermont-Ferrand

<sup>4</sup>Nurse Anesthetist School, CHU Clermont-Ferrand

Correspondence to: Raïko Blondonnet at [rblondonnet@chu-clermontferrand.fr](mailto:rblondonnet@chu-clermontferrand.fr)

URL: <https://www.jove.com/video/58554>

DOI: [doi:10.3791/58554](https://doi.org/10.3791/58554)

Keywords: Medicine, Issue 140, Cell culture, sevoflurane, isoflurane, halogenated gases, alveolar epithelial cells, lung, ARDS, air-liquid interface, chromatography

Date Published: 10/23/2018

Citation: Blondonnet, R., Paquette, B., Richard, D., Bourg, R., Laplace, G., Segurel, R., Pouvelle, H., Belville, C., Blanchon, L., Godet, T., Constantin, J.M., Bazin, J.E., Sapin, V., Jabaudon, M. *In Vitro* Method to Control Concentrations of Halogenated Gases in Cultured Alveolar Epithelial Cells. *J. Vis. Exp.* (140), e58554, doi:10.3791/58554 (2018).

## **Abstract**

Acute respiratory distress syndrome (ARDS) is a syndrome of diffuse alveolar injury with impaired alveolar fluid clearance and severe inflammation. The use of halogenated agents, such as sevoflurane or isoflurane, for the sedation of intensive care unit (ICU) patients can improve gas exchange, reduce alveolar edema, and attenuate inflammation during ARDS. However, data on the use of inhaled agents for continuous sedation in the ICU to treat or prevent lung damage is lacking. To study the effects of halogenated agents on alveolar epithelial cells under "physiologic" conditions, we describe an easy system to culture cells at the air-liquid interface and expose them to halogenated agents to provide precise controlled "air" fractions and "medium" concentrations for these agents. We developed a sealed air-tight chamber in which plates with human alveolar epithelial immortalized cells could be exposed to a precise, controlled fraction of sevoflurane or isoflurane using a continuous gas flow provided by an anesthetic machine circuit. Cells were exposed to 4% of sevoflurane and 1% of isoflurane for 24 hours. Gas mass spectrometry was performed to determine the concentration of halogenated agents dissolved in the medium. After the first hour, the concentrations of sevoflurane and isoflurane in the medium were 251 mg/L and 25 mg/L, respectively. The curves representing the concentrations of both sevoflurane and isoflurane dissolved in the medium showed similar courses over time, with a plateau reached at one hour after exposure.

This protocol was specifically designed to reach precise and controlled concentrations of sevoflurane or isoflurane *in vitro* to improve our understanding of mechanisms involved in epithelial lung injury during ARDS and to test novel therapies for the syndrome.

## **Video Link**

The video component of this article can be found at <https://www.jove.com/video/58554/>

## **Introduction**

Acute respiratory distress syndrome (ARDS) is a clinical syndrome characterized by diffuse alveolar injury, lung edema, and hypoxemic respiratory failure. Although ARDS represents more than 10% of intensive care unit (ICU) admissions and nearly 25% of ICU patients requiring mechanical ventilation, it is still an under-recognized challenge for clinicians, with a hospital mortality rate of 35–45%<sup>1</sup>. Despite intense research, the identification of an effective ARDS pharmacologic therapy or prevention has failed to date. Two major features contribute to mortality in ARDS: impaired alveolar fluid clearance (AFC) (*i.e.*, the altered resorption of alveolar edema fluid from distal lung airspaces) and severe inflammation<sup>2</sup>. Since ARDS mortality remains high, current initiatives should also include primary prevention; however, a key challenge is to identify at-risk patients in whom ARDS is likely to develop and who would benefit if ARDS were prevented.

Volatile halogenated anesthetics, such as sevoflurane and isoflurane, are widely used to provide general anesthesia in the operating room. Worldwide, more than 230 million patients undergoing major surgery each year require general anesthesia and mechanical ventilation<sup>3</sup>, and postoperative pulmonary complications adversely affect clinical outcomes and healthcare utilization<sup>4</sup>. The use of sevoflurane instead of propofol was associated with improved lung inflammation in patients undergoing thoracic surgery and significant decreases in adverse events, such as ARDS and postoperative pulmonary complications<sup>5</sup>. Similarly, pretreatment with isoflurane had protective effects on respiratory mechanics, oxygenation, and hemodynamics in experimental animal models of ARDS<sup>6,7</sup>. Although further studies are warranted to address the impact of inhaled agents on outcomes in noncardiac surgery, a similar decrease in pulmonary complications has been recently observed in a meta-

analysis, demonstrating that inhaled anesthetic agents—as opposed to intravenous anesthesia—are significantly associated with a reduction in mortality for cardiac surgery<sup>8</sup>.

Specific prospective data about the use of volatile agents for the sedation of ICU patients to prevent or treat lung damage is lacking. However, several trials now support the efficacy and safety of inhaled sevoflurane for the sedation of ICU patients, and preclinical studies have shown that inhaled sevoflurane and isoflurane<sup>7,9</sup> improve gas exchange, reduce alveolar edema, and attenuate inflammation in experimental models of ARDS. Additionally, sevoflurane mitigates type II epithelial cell damage<sup>10</sup>, whereas isoflurane maintains the integrity of the alveolar-capillary barrier through modulation of tight junction protein<sup>11</sup>. However, further studies are needed to verify to what extent the experimental evidence of organ protection from inhaled sevoflurane and isoflurane could be translated to humans. A first single-center randomized controlled-trial (RCT) from our group found that early use of inhaled sevoflurane in patients with ARDS was associated with improved oxygenation, reduced levels of some pro-inflammatory markers, and reduced lung epithelial damage, as assessed by the levels of the soluble form of the receptor for advanced glycation end-products (sRAGE) in plasma and alveolar fluid<sup>12</sup>.

Taken together, the beneficial effects of sevoflurane and isoflurane on lung injury could point to multiple biological pathways or functional processes that are dependent on the RAGE pathway, namely alveolar fluid clearance (AFC), epithelial injury, translocation of nuclear factor (NF)- $\kappa$ B, and macrophage activation. In addition, sevoflurane may influence the expression of the RAGE protein itself. Since previous research by our research team and others supports pivotal roles for RAGE in alveolar inflammation and lung epithelial injury/repair during ARDS, we designed an experimental model to provide a translational understanding of the mechanisms of sevoflurane in lung injury and repair<sup>13,14,15</sup>. The *in vitro* effects of sevoflurane and isoflurane were investigated in a novel human alveolar epithelial primary cell line specifically designed to study the air-blood barrier of the peripheral lung, hAELVi (human Alveolar Epithelial LentiVirus immortalized), with alveolar type I-like characteristics including functional tight junctions<sup>16</sup>.

While preparing the design of our *in vitro* investigations (e.g., cultures of alveolar epithelial cells at the air-liquid interface with exposure to "inhaled" sevoflurane or isoflurane, we understood from previously published studies that fractions of sevoflurane have only been assessed in the "air" interface<sup>17,18,19</sup> using standard monitors (similar to those used in a clinical setting). Halogenated agent concentrations were usually chosen according to the minimum alveolar concentration (MAC) values (e.g., in humans, for sevoflurane, 0.5, 1.1, and 2.2 vol%, representing 0.25, 0.5, and 1 MAC, respectively; for isoflurane, 0.6, 0.8, and 1.3 vol% representing 0.25, 0.5, and 1 MAC, respectively)<sup>20</sup>. Indeed, sevoflurane and isoflurane concentrations have never been investigated in the culture medium itself, thus limiting the validity of previous experimental models/instruments. Furthermore, most experiments used an anaerobic jar that was sealed after the air mix containing sevoflurane had been flushed inside. As our goal was to study alveolar epithelial cells under "physiologic" conditions, we believed that such an anaerobic state may not be optimal and would not be compatible with long experimental durations. Therefore, we developed our own system to culture cells at the air-liquid interface and expose them to halogenated agents (sevoflurane and isoflurane) with the aim of providing precise controlled "air" fractions and "medium" concentrations for these agents. In our opinion, this experimental step, which has not been reported to date in the literature, is mandatory prior to any further *in vitro* investigations of sevoflurane and isoflurane.

## Protocol

### 1. Culture of Alveolar Epithelial Cells (hAELVi)

#### 1. Thawing

1. Pipette 4 mL of cultivation ready-to-use human alveolar epithelial (huAEC) medium in a 15 mL plastic tube and quickly thaw the vial in a preheated water bath (37 °C).
2. Transfer the thawed cell suspension to a 15 mL plastic tube containing 4 mL of the medium before centrifuging the tube at 200 x g for 5 min.
3. Aspirate the supernatant and resuspend the cell pellet with 5 mL of the cultivation medium. Then, transfer the cells to a T25 flask.
4. Cultivate the cells under standard conditions (5% CO<sub>2</sub>, 95% humidified air, 37 °C)

#### 2. Splitting

1. Check the status of the cells microscopically. When the cells are 80-90% confluent, split the cells, following steps 1.2.2 through 1.2.10.
2. Aspirate the cultivation media of the cells with a sterile pipette.
3. Wash the cells once with 4 mL of Dulbecco's Phosphate Buffered Saline (DPBS) and aspirate the DPBS.
4. Add 1 mL of Trypsin/EDTA solution (TE) to the cells prior to incubating at 37 °C for 3 min until the cells start to detach; check for detachment under a microscope.
5. Resuspend the cells with 2 mL of the culture medium and centrifuge the cells at 200 x g for 5 min. Then, aspirate the supernatant and resuspend the cell pellet again with 3 mL of the cultivation medium.
6. Use the trypan blue dye exclusion assay to determine the viability of the cells. Take 30  $\mu$ L of the cell suspension and add 30  $\mu$ L of a 0.4% solution of trypan blue in a tube. After that, effectively mix the solution 3-5 times using a 100  $\mu$ L pipette. Take 10  $\mu$ L of the solution and put it under the coverslip of the hemocytometer.
7. Count both the total number of viable cells and the number of dead cells (blue) in the areas of the hemocytometer. Then, calculate the cell viability [%] using the equation: cell viability = 100 - (100 / total number of cells x number of dead cells)
8. Transfer the aliquot with the resuspended cell pellet to a new cell culture T25 flask or a 6 well-plate. Add the cultivation medium to the cells to achieve a total of 5 mL of cultivation medium in the T25 flask, or 1 mL for each well of the 6 well-plate.
9. Cultivate the cells in an incubator under standard conditions (5% CO<sub>2</sub>, 95% humidified air, 37 °C)
10. Once the cells are completely confluent, they are ready for the experiment.

## 2. Preparation of an Air-tight Chamber

NOTE: The construction plan for the air-tight chamber is depicted in **Figure 1**.

1. Use a hermetic polypropylene box with a capacity of 6.5 L. The length, width, and height are 30 x 20 x 15.5 cm, respectively. Please note, the volume of the box is lower than the theoretical volume because of the rounded corners.
2. Drill a 2.5 cm diameter hole on the bottom side of the lateral wall.
3. Insert a corrugated tube with a green mark, which will serve as the gas-air mixture input pipe, and seal it with silicon.
4. Drill a second 2.5 cm diameter hole on the top side of the opposite lateral wall.
5. Insert another corrugated tube with a red mark and connect it with a charcoal filter, which will serve as the gas-air mixture output pipe, and seal it with silicon.
6. Drill a tight 4 mm diameter hole at the center of the mean wall of the box.
7. Insert short infusion tubing that is connected at a manifold with a rotating male luer-lock, which will be plugged into a gas analyzer, and seal it with silicon.
8. Place a digital thermometer/hygrometer inside the air-tight chamber.

## 3. Expose Alveolar Epithelial Cells to Halogenated Agents (Sevoflurane and Isoflurane)

NOTE: A schematic drawing of the device is depicted in **Figure 2**.

CAUTION: Although animal studies have revealed no evidence of fetal harm or impaired fertility, and a very small study during cesarean sections did not show any untoward effects on the mother or fetus, the safety of using halogenated agents (e.g., sevoflurane or isoflurane) during labor and delivery has not been demonstrated to date. Furthermore, no controlled data has been collected during human pregnancies. Therefore, performing experiments using sevoflurane or isoflurane while pregnant should be strongly discouraged.

1. Work under a laboratory extractor hood.
2. Customize an anesthetic machine circuit to switch the gas line of nitrous oxide by carbon dioxide (CO<sub>2</sub>).
3. Plug the air-tight chamber with the green-marked, corrugated tube into the customized anesthetic machine circuit. Insert a heated humidifier (such as those used on ICU ventilators) into the pipe between the anesthetic machine and the air-tight chamber to warm the gas flow mixture to approximately 37 °C.
4. Install the air-tight chamber on a hot plate, providing a heating plate temperature of 37°C.
5. Put the 6 well-plate containing the hAELVI cells into the air-tight chamber and seal the lid.
6. Regulate the gas flow rates (*i.e.*, the mixture of air and CO<sub>2</sub>) to quickly obtain the standard conditions, defined as 5% of CO<sub>2</sub> and 95% of humidified air.
7. Open the halogenated agent evaporator and choose the percentage desired (in the present study, the tested concentrations of sevoflurane and isoflurane were 4% and 1%, respectively).
8. Note the composition of gas mixture and the sevoflurane or isoflurane concentration as measured by an external gas analyzer and displayed on the screen.
9. Once the target values are achieved, reduce the fresh gas flow rate to 1 L/min.
10. The air-tight chamber can be maintained with this gas flow rate as long as necessary for the experiment.

## 4. Measure Sevoflurane or Isoflurane by Chromatography

1. Preparation of samples
  1. At different time points, very briefly open the chamber to take out the studied samples (in the present study, we used a 6 well-plate) and close the lid. Keep the other samples in the box. Then, aspirate 1 mL of the medium contained in each sample with a multi-volume adjustable micropipette.
  2. Put the medium into 10 mL headspace chromatography vials, which should be screwed hermetically tight with a Teflon-sealed cap. Freeze the chromatography vials at -20 °C if you do not use them immediately.
  3. Prepare a stock solution of sevoflurane and another stock solution of isoflurane, both at 50 g/L in methanol. Simultaneously, prepare a stock solution of chloroform (internal standard, IS) at 2 g/L in methanol. Store all standard solutions at -20 °C.
  4. Prepare working solutions of sevoflurane and isoflurane at 50, 500, and 5000 mg/L in ultrapure water/dimethyl sulfoxide (50/50; v/v). For internal standardization, the working solution is fixed at 100 mg/L in methanol.

### 2. Analysis of cellular samples

NOTE: The extraction procedure is based on the previously validated method of gas chromatography and mass spectrometry from Bourdeaux *et al.*<sup>1</sup> and uses the same parameters of sensibility and specificity. Sevoflurane and chloroform (IS) were used in this protocol, and isoflurane was associated with the multiparametric analytical method. Briefly, for mass spectrometry acquisition, the method was developed after pure solution injection. Then, m/z was confirmed with reference standards and literature data. Three m/z were selected for each analyte (except IS): one m/z for quantification (the most abundant and the higher), for which abundance was calculated by integrating the area under the curve for quantification, and two m/z for confirmation. Using three m/z, analytes could be specifically identified because all m/z had the same retention time, as well as because all m/z amounts (relative of ion confirmation vs. quantification) were the same in the pure standard and in all samples. With this acquisition mode, analytes could be identified and quantified with good specificity.

1. Construct a calibration 8-point curves with the concentration ranges of 0.5–400 mg/L and multiple quality controls (0.5, 1, 5, 10, 20, 75, 200, and 400 mg/L).

NOTE: To validate each calibration, four quality controls were used: the lower limit of quantification (C1 = 0.5 mg/L), two intermediate levels (C2 = 20 mg/L and C3 = 75 mg/L) and the final level (C4 at 400 mg/L; upper level of quantification). All standards and controls

were analyzed in cultured cell matrices to avoid the matrix effect. For each calibration curve, a blank matrix was analyzed to validate that there was no interference with cultured cellular and internal standards.

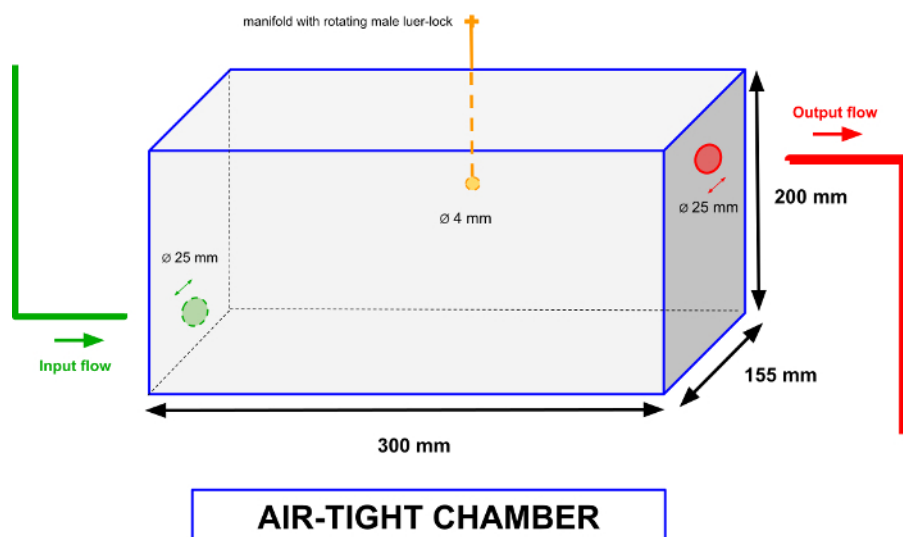
2. Prepare a stock solution of sevoflurane and another stock solution of isoflurane, both at 50 g/L in methanol. Simultaneously, prepare a stock solution of chloroform (internal standard, IS) at 2 g/L in methanol. Store all standard solutions at -20 °C. Then, prepare working solutions of mixed sevoflurane/isoflurane at 50, 500, and 5000 mg/L in ultrapure water / dimethyl sulfoxide (50/50; v/v). For IS, the working solution is diluted at 100 mg/L in methanol.
3. For calibration curves and controls, prepare the samples by spiking 50  $\mu$ L of IS (100 mg/L) into 1 mL of the cellular sample matrices.
4. Prepare sample solutions with 200  $\mu$ L of saturated sodium chloride water solution in a 10 mL headspace tube, screwed hermetically tight with a Teflon-sealed cap.
3. Gas chromatography and mass spectrometry
  1. For sample analyses, use headspace injections in a gas chromatography, coupled with the mass detection method.
  2. Carry headspace tubes for 10 min at 80 °C with a heater shaker. Then, withdraw and inject 1.5 mL of the gas sample into the gas chromatograph. Set the parameter of the injector at 260 °C with a split flow at 100 mL/min for 2 min at the start of the chromatography run.
  3. Use Split/splitless injector with a carrier mode-programmed pressure. First, keep the gas pressure at 40 kPa for 0.15 min. Then, increase the rate program pressure to 150 kPa at 125 kPa/min before setting a rate of 16 kPa/min to 300 kPa pressure for 5 min.
  4. Simultaneous to the injection, start with an oven temperature of 60 °C for 1 min and increase until a temperature of 140 °C is reached at a rate of 20 °C/min. Then, increase the temperature again until 250 °C is achieved. The total time of the run is 7 min.
  5. Carry out the chromatography separation using a fused-silica capillary column (30 m x 1.4  $\mu$ m, 0.25 mm ID). Perform mass experiments with a single ion monitoring (SIM) condition, and monitor ion quantification m/z 181, and ion qualifications m/z 151 and 51 simultaneously at a retention time (RT) of 2.30 min for sevoflurane, m/z 149 (ion quantification), m/z 115 and 87 (ion qualifications) for isoflurane at RT 2.8 min, and m/z 83 (ion quantification) for chloroform at RT 3.70 min.
  6. Determine the concentrations of sevoflurane and isoflurane in the cell culture by their area ratios to that of the IS using a weight quadratic fit. The lower limit of quantification (LLOQ) for sevoflurane and isoflurane was at 0.5 mg/L and the upper limit of quantification (ULOQ) was 400 mg/L.

## Representative Results

The concentrations of the sevoflurane and isoflurane, which dissolved in the medium over time, are reported in **Table 1** and **Table 2**, respectively.

The courses of the sevoflurane and isoflurane concentrations in the medium were similar over time. Immediately after the required concentration of halogenated agent was set, concentrations rose over the first hour. A plateau was then reached, which persisted until the administration of the halogenated agent was stopped. After administration interruption, concentrations decreased within one hour (**Figure 3**).

After the first hour, the median concentrations of sevoflurane and isoflurane in the medium were 251 mg/L and 25 mg/L, respectively. No significant difference was found between the different experiments.



**Figure 1: Construction plan of the air-tight chamber** [Please click here to view a larger version of this figure.](#)

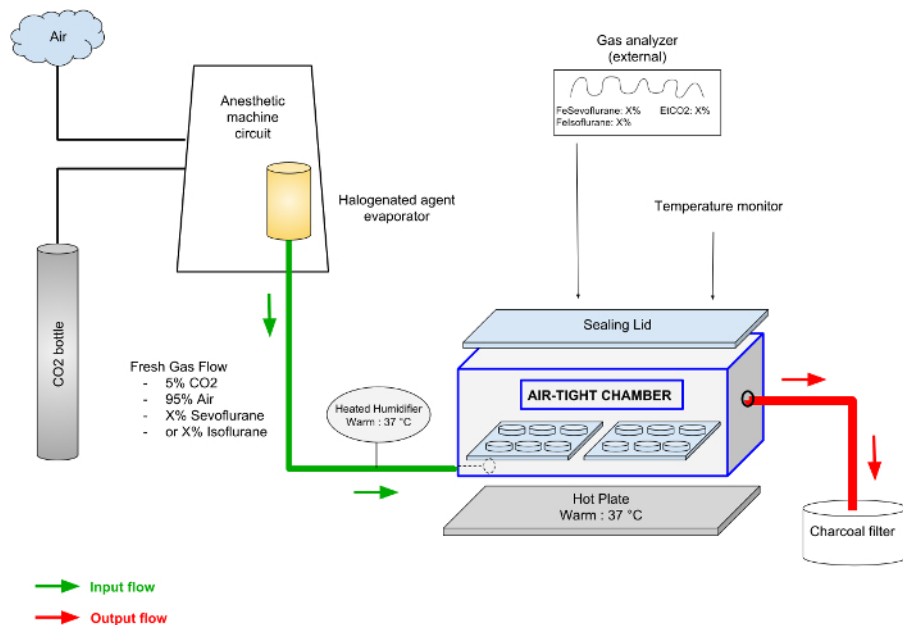
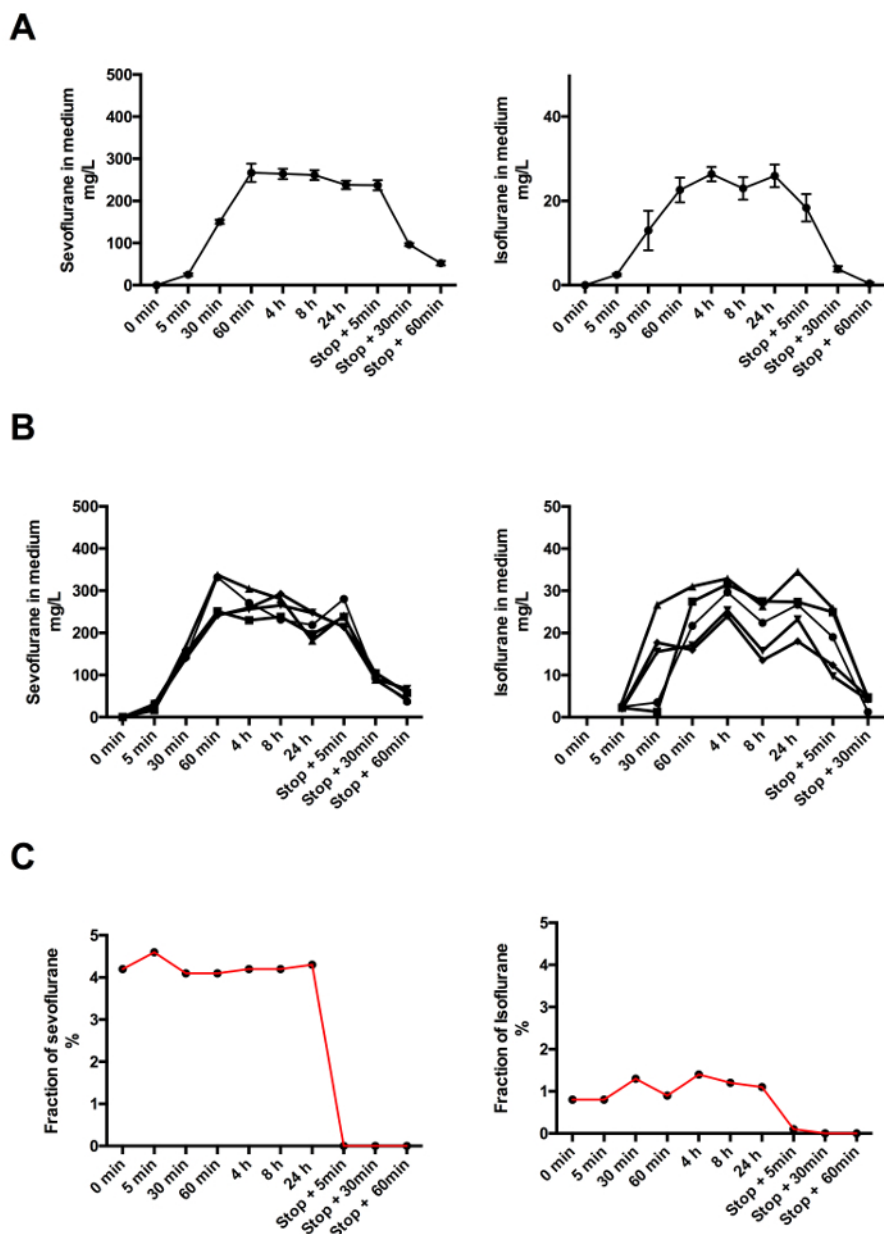


Figure 2: Schematic drawing of the device [Please click here to view a larger version of this figure.](#)



**Figure 3: Concentration of sevoflurane (n = 5) and isoflurane (n = 5) over time. A)** Concentration of halogenated agent over time. Values are expressed in mg/L. Values are expressed in mean and SEM. **B)** Concentration of halogenated agent over time for each experiment. Value are expressed in mg/L. **C)** Fraction of halogenated agent over time in the air-tight chamber measured by the gas analyzer. Values are expressed in percentages. [Please click here to view a larger version of this figure.](#)

Time	Concentration of sevoflurane in the medium		Fraction of sevoflurane
	(mg/L)		in the air-tight chamber
	Median	IC	(%)
5 min	27	[19 - 31]	4.6
30 min	152	[142 - 152]	4.1
1 h	251	[243 - 332]	4.1
4 h	259	[256 - 271]	4.2
8h	265	[237 - 280]	4.2
24 h (stop)	218	[196 - 247]	4.3
Stop + 5 min	237	[214 - 241]	0
Stop + 30 min	92	[91 - 104]	0
Stop + 1 h	57	[42 - 58]	0

**Table 1: Concentrations of sevoflurane dissolved in the medium over time.** Numerical data are expressed as a median value with interquartile range for the concentration and as percentage for the fraction. IQR (for interquartile range)

Time	Concentration of isoflurane in the medium		Fraction of isoflurane
	(mg/L)		in the air-tight chamber
	Median	IC	(%)
5min	2	[2 - 2.5]	0.8
30min	16	[4 - 18]	1.3
1h	22	[18 - 27]	0.9
4h	30	[25 - 31]	1.4
8h	22	[15 - 26]	1.2
24h (stop)	26	[23 - 27]	1.1
Stop + 5min	19	[12 - 25]	0
Stop + 30min	4	[4 - 4]	0
Stop + 1h	1	[0.8 - 1]	0

**Table 2: Concentrations of isoflurane dissolved in the medium over time.** Numerical data are expressed as a median value with interquartile range for the concentration and as percentage for the fraction. IQR (for interquartile range)

## Discussion

Our protocol describes an easy method to expose cells to a precise fraction of a halogenated anesthetic agent, such as sevoflurane or isoflurane. Furthermore, we report here—for the first time—a rigorous correlation between both the gas fraction and the concentration of sevoflurane and isoflurane inside the culture medium itself. This fundamental step now allows us to safely use our air-tight chamber to study the effects of these halogenated agents in a cultured monolayer of human alveolar epithelial cells.

Currently, most research teams studying the effects of sevoflurane in alveolar cells use a jar that is first saturated with halogenated gas and then sealed. In this case, sevoflurane may be metabolized, and it could be speculated that the fraction of volatile agent may decrease linearly over time, leading to an unstable gas concentration. However, the correlation between the gas fraction of sevoflurane and its concentration in the culture medium is not clearly reported in the literature. Usually, the concentration of sevoflurane used in these experiments is chosen based on a simple relationship between the gas fraction and the MAC. MAC was introduced in 1965 and is the concentration of a vapor in the lungs that is needed to prevent a motor response (movement) in 50% of subjects in response to a surgical stimulus (pain)<sup>22</sup>. MAC is used to compare the strength, or potency, of anesthetic vapors. In ICU patients, MAC is correlated to FeSevo and the clinical Richmond Assessment Agitation-Sedation Scale (RASS)<sup>23</sup>. Although it is a useful indicator in daily clinical practice, the relevance of this parameter has never been investigated in the setting of experimental *in vitro* research. In our protocol, using chromatography analyses of the medium, we determined the precise correlation between the sevoflurane contained in the gas fraction and the sevoflurane dissolved into the medium. With this method, the specific effect of a volatile agent is expressed according to the real concentration in the medium rather than based on the approximation of a clinical effect. This important element allows the study of the specific effect of a precise concentration of a halogenated agent on cells growing in a medium, in order to compare the effects of different concentrations of inhaled agents. Furthermore, as the air-tight chamber is very easy to use, this method allows researchers to replicate the experiment with precision.

Another important point that may preclude the use of the correlation between gas fraction and MAC in experimental research is that a halogenated agent has low solubility in blood (blood/gas partition coefficient at 37°C = 0.63 to 0.69 for sevoflurane). A minimal quantity of sevoflurane is mandated to dissolve in the blood before the pressure in the alveoli achieves equilibrium with the pressure in the arterial. Thus, during the induction of anesthesia, the alveolar (end-tidal) concentration (AF, alveolar fraction) of sevoflurane rapidly increases around the



inspired concentration (FI, inspired fraction). However, *in vitro* culture conditions do not allow such mechanisms, and usual cell media mainly consist of aqueous solutions. Furthermore, the solubility coefficient between water/gas (partition coefficient at 37°C = 0.36 for sevoflurane) is lower than between blood and gas, underlying the critical importance of performing chromatography analyses.

Additionally, when a sealed jar is used, the atmospheric oxygen in the jar is absorbed by the cells with the simultaneous generation of carbon dioxide. This effect is probably insignificant in short experimental procedures, but for longer experimental durations, cells that are deprived of oxygen would switch to an anaerobic metabolism; this change in metabolism may induce a certain degree of bias in experimental analyses. In contrast to the sealed jar, when using our air-tight chamber, both oxygen and halogenated agent flows are adjustable over time to maintain the targeted level. This major characteristic of our protocol therefore allows the design of *in vitro* experiments for long time periods (e.g., more than one day), making it an interesting tool to study the cellular mechanisms involved in lung epithelial injury and repair over time, especially when halogenated agents are used. Indeed, the effects of inhaled anesthetic agents on lung cells or tissue during alveolar injury remain poorly investigated to date while this alternative therapy seems to show very encouraging results<sup>12</sup>.

However, there are limitations to this technique. First, an anesthetic machine circuit is needed to provide oxygen, carbon dioxide, and halogenated agent gas flows. Using such a device is mandatory to set the flow rate and maintain stable concentrations over time. Second, to sample the medium prior to chromatography analyses, the air-tight chamber is briefly opened, which induces a transient decrease in the gas concentrations. As we use an anesthetic machine circuit, gas flows are thereafter increased until expected concentrations are achieved again on the gas analyzer. Third, we have measured the concentration in the medium for only one fraction of each halogenated agent, chosen *a priori* based on previous study. Fourth, to stabilize the cell medium to the growth of alveolar epithelial cells, we need to use carbon dioxide at a concentration of 5%. Indeed, no anesthetic machine circuit provides such a concentration of carbon dioxide. Therefore, the anesthetic machine circuit needs to be customized to allow the connection of carbon dioxide gas flow in place of nitrous oxide. Such a connection should be used exclusively in the setting of experimental research and should cautiously be unplugged after each experiment. Furthermore, to avoid any risk for humans, we invite researchers to use a devoted anesthetic machine circuit to perform this protocol and not to use a machine dedicated to clinical anesthesia.

The main advantages of this technique are that it is relatively inexpensive and very easy to adopt, even when researchers have never manipulated an airtight chamber before. Moreover, with our protocol, the results of dissolved sevoflurane and isoflurane concentrations are reproducible, which represents a major quality criterion for experimental research. In addition, our system could allow for the study of other volatile halogenated agents, such as desflurane. Indeed, a simple change of the type of gas evaporator device would be sufficient in this case. Similarly, our system could provide a means to study the concentrations of sevoflurane or isoflurane dissolved in any type of medium with different solubilities, such as water, blood, or oil.

Our experiment represents a fundamental step that is part of a larger project designed to test the hypothesis that sevoflurane and isoflurane may exert beneficial effects on lung injury, inflammation, and AFC through RAGE-mediated pathways. A primary culture of human alveolar epithelial cells will be used for mechanistic investigations of transepithelial fluid transport, channel-specific fluid transport (e.g., using pharmacological antagonism), epithelial paracellular permeability, wound repair, cell migration and proliferation, with or without a halogenated anesthetic agent (sevoflurane or isoflurane), alone or combined with cytomix (*in vitro* model of alveolar injury)<sup>24</sup>.

In conclusion, this protocol was specifically designed to reach precise and controlled concentrations of sevoflurane or isoflurane *in vitro* to improve our understanding of mechanisms involved in epithelial lung injury during ARDS and to test novel therapies for this frequent and life-threatening syndrome.

## Disclosures

The authors declare that they have no competing financial interests.

## Acknowledgements

The authors acknowledge the Auvergne Regional Council ("Programme Nouveau Chercheur de la Région Auvergne" 2013) and the French Agence Nationale de la Recherche and the Direction Générale de L'Offre de Soins ("Programme de Recherche Translationnelle en Santé" ANR-13-PRTS-0010) for the grants. The funders had no influence in the study design, conduct, and analysis or in the preparation of this article.

## References

1. Bellani, G., Laffey, J. G., *et al.* Epidemiology, Patterns of Care, and Mortality for Patients With Acute Respiratory Distress Syndrome in Intensive Care Units in 50 Countries. *JAMA: T Journal of the American Medical Association*. **315**, (8)788-800 (2016).
2. Thompson, B. T., Chambers, R. C., & Liu, K. D. Acute Respiratory Distress Syndrome. *The New England Journal of Medicine*. **377**, (6)562-572 (2017).
3. Weiser, T. G., Regenbogen, S. E., *et al.* An estimation of the global volume of surgery: a modelling strategy based on available data. *The Lancet*. **372**, (9633)139-144 (2008).
4. Khuri, S. F., Henderson, W. G., *et al.* Determinants of long-term survival after major surgery and the adverse effect of postoperative complications. *Annals of Surgery*. **242**, (3)326-41; discussion 341-3 (2005).
5. De Conno, E., Steurer, M. P., *et al.* Anesthetic-induced improvement of the inflammatory response to one-lung ventilation. *Anesthesiology*. **110**, (6)1316-1326 (2009).
6. Fu, H., Sun, M., & Miao, C. Effects of different concentrations of isoflurane pretreatment on respiratory mechanics, oxygenation and hemodynamics in LPS-induced acute respiratory distress syndrome model of juvenile piglets. *Experimental Lung Research*. **41**, (8)415-421 (2015).



7. Reutershan, J., Chang, D., Hayes, J. K., & Ley, K. Protective effects of isoflurane pretreatment in endotoxin-induced lung injury. *Anesthesiology*. **104**, (3)511-517 (2006).
8. Uhlig, C., Bluth, T., *et al.* Effects of Volatile Anesthetics on Mortality and Postoperative Pulmonary and Other Complications in Patients Undergoing Surgery: A Systematic Review and Meta-analysis. *Anesthesiology: The Journal of the American Society of Anesthesiologists*. **124**, (6)1230-1245 (2016).
9. Li, Q. F., Zhu, Y. S., Jiang, H., Xu, H., & Sun, Y. Isoflurane preconditioning ameliorates endotoxin-induced acute lung injury and mortality in rats. *Anesthesia and Analgesia*. **109**, (5)1591-1597 (2009).
10. Voigtsberger, S., Lachmann, R. A., *et al.* Sevoflurane ameliorates gas exchange and attenuates lung damage in experimental lipopolysaccharide-induced lung injury. *Anesthesiology*. **111**, (6)1238-1248 (2009).
11. Englert, J. A., Macias, A. A., *et al.* Isoflurane Ameliorates Acute Lung Injury by Preserving Epithelial Tight Junction Integrity. *Anesthesiology*. **123**, (2)377-388 (2015).
12. Jabaudon, M., Boucher, P., *et al.* Sevoflurane for Sedation in ARDS: A Randomized Controlled Pilot Study. *American Journal of Respiratory and Critical Care Medicine*. (2016).
13. Blondonnet, R., Audard, J., *et al.* RAGE inhibition reduces acute lung injury in mice. *Scientific Reports*. **7** (1) 7208 (2017).
14. Jabaudon, M., Blondonnet, R., *et al.* Soluble Receptor for Advanced Glycation End-Products Predicts Impaired Alveolar Fluid Clearance in Acute Respiratory Distress Syndrome. *American Journal of Respiratory and Critical Care Medicine*. **192**, (2)191-199 (2015).
15. Jabaudon, M., Blondonnet, R., *et al.* Soluble Forms and Ligands of the Receptor for Advanced Glycation End-Products in Patients with Acute Respiratory Distress Syndrome: An Observational Prospective Study. *PloS One*. **10**, (8)e0135857 (2015).
16. Kuehn, A., Kletting, S., *et al.* Human alveolar epithelial cells expressing tight junctions to model the air-blood barrier. *ALTEX*. **33**, (3)251-260 (2016).
17. Yue, T., Roth Z'raggen, B., *et al.* Postconditioning with a volatile anaesthetic in alveolar epithelial cells *in vitro*. *The European Respiratory Journal: Official Journal of the European Society for Clinical Respiratory Physiology*. **31**, (1)118-125 (2008).
18. Suter, D., Spahn, D. R., *et al.* The immunomodulatory effect of sevoflurane in endotoxin-injured alveolar epithelial cells. *Anesthesia and Analgesia*. **104**, (3)638-645 (2007).
19. Schl pfer, M., Leutert, A. C., Voigtsberger, S., Lachmann, R. A., Booy, C., & Beck-Schimmer, B. Sevoflurane reduces severity of acute lung injury possibly by impairing formation of alveolar oedema. *Clinical and Experimental Immunology*. **168**, (1)125-134 (2012).
20. Nickalls, R. W. D., & Mapleson, W. W. Age-related iso-MAC charts for isoflurane, sevoflurane and desflurane in man. *British Journal of Anaesthesia*. **91**, (2)170-174 (2003).
21. Bourdeaux, D., Sautou-Miranda, V., *et al.* Simple assay of plasma sevoflurane and its metabolite hexafluoroisopropanol by headspace GC-MS. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*. **878**, (1)45-50 (2010).
22. Eger, E. I., Saidman, L. J., & Brandstater, B. Minimum alveolar anesthetic concentration. *Anesthesiology*. **26**, (6)756-763 (1965).
23. Perbet, S., Fernandez-Canal, C., Pereira, B., Cardot, J. M., Bazin, J. E., & Constantin, J. M. Evaluation of Richmond Agitation Sedation Scale According To Alveolar Concentration of Sevoflurane During a Sedation With Sevoflurane in Icu Patients. *Intensive Care Medicine Experimental*. **3**, (Suppl 1) (2015).
24. Goolaerts, A., Pell n-Randrianarison, N., *et al.* Conditioned media from mesenchymal stromal cells restore sodium transport and preserve epithelial permeability in an *in vitro* model of acute alveolar injury. *American Journal of Physiology. Lung Cellular and Molecular Physiology*. **306**, (11)L975-85 (2014).